

REMARKS

The undersigned wishes to thank Examiner Nolan for granting a telephonic interview to the undersigned on September 20, 2005 to advance prosecution of the application.

Claims 1-13 were pending in the subject application. Applicants have amended claim 1 to clarify the claimed subject matter as suggested by Examiners Nolan and Szperka. No new matter is being added through this amendment. Applicants respectfully request entry of this amendment so that claims 1-13 will be pending.

Oath/Declaration

In the Office Action of April 1, 2005, the Examiner had objected to the declaration as allegedly failing to comply with 37 CFR 1.67(a). Applicants submitted a new declaration with the response filed on August 3, 2005. Because a defective declaration was not cited as a ground of rejection in the advisory action mailed on August 29, 2005, Applicants infer that the new declaration has overcome the previous objection. Applicants, however, request respectfully request confirmation.

Claim Rejections under 35 USC 112 1st Paragraph-Enablement

The Office Action maintains its rejection of claims 1-3 as allegedly failing to comply with the enablement requirement.

The previous Office Action alleged that Veldman *et al.* teaches that both healthy and PV patients have reactive T-cells to the claimed peptide, and therefore concludes that the claimed peptide is not causative or therapeutic for PV.

Applicants traversed this rejection in the previous communication, because the peptides of Veldman and the peptide of claim 1 were not the same peptide:

Applicants respectfully traverse, because none of the peptides used in Veldman are identical to the claimed peptide. The least divergent peptide in Veldman spans residues 189-205 of desmoglein 3, while the claimed peptide spans residues 186-

204. The two peptides are not the same peptide. In fact, they share only 84% (16/19 residues) amino acid identity. Accordingly, the teachings of Veldman do not apply to the claimed peptide because they describe different peptides. Furthermore, the Office Action has failed to provide a rationale why the alleged teachings of one Desmoglein 3 peptide apply to different Desmoglein 3 peptides. The Office Action has failed to justify its underlying assumption that two peptides having 84% identity must have identical biological properties *in vivo*. Since the peptides of Veldman are different from the claimed peptide, Veldman fails to undermine the therapeutic effectiveness the properties of the claimed peptide. Should the this rejection be maintained in a future Office Communication, applicants respectfully request that the basis for this assumption be made of record so that prosecution of the application can move forward. (see page 5, 2nd paragraph).

The advisory action concedes that the peptides of Veldman do not have the sequence of SEQ ID NO:1, but counters that claim 1 encompasses not only a peptide having the exact amino acid sequence of SEQ ID NO:1 but also any fragments of SEQ ID NO:1. In particular, the advisory action interprets the phrase “of *an* amino acid sequence” as encompassing multiple sequences, due to the article “an”. In response, Applicants have amended claim 1 to replace the article “an” with “the” so that claim 1 is not read to encompass fragments of SEQ ID NO:1.

Applicants respectfully submit the peptide of claim 1 is not described in Veldman et al. Therefore, Veldman is irrelevant to the patentability of the claims and does not undermine the uses of the claimed peptide.

(II) Veldman Does not Teach that Dsg3 Peptides Have No Causal Role in PV

Even if the peptides taught by Veldman were the same as the peptide of claim 1, which applicants have shown above is not the case, Veldman fails to teach or suggest that the peptides of the invention would be ineffective in treating PV.

In its logic, the Office Action of April 1, 2005 confuses the *necessity* of Th1 cell responses to a Dsg3 antigen with the *sufficiency* of such responses in causing PV. The Office Action reasons that if Th1 cell responses to Dsg3 are not sufficient to cause PV (and they are allegedly not

sufficient because healthy subjects having those Th1 cell responses do not have PV), then the Th1 responses must play no role in causing PV. But that conclusion does not logically follow: just because they might not sufficient, on their own, to cause PV, it does not mean that they are not a necessary and integral requirement for developing PV.

The Office Action's logic is equivalent to saying that because patients suffering from an autoimmune disease and normal subjects both have an immune system, then the immune system cannot contribute to the autoimmune disease, or equivalent to saying that because patients suffering from an autoimmune diseases and normal subjects both have an immune system, suppressing the immune system cannot be therapeutic to the autoimmune-disease patient. Both of these statements are clearly wrong, as autoimmune diseases are caused by a malfunctioning immune system and suppression of the immune system is used to treat autoimmune diseases.

The last sentence of the abstract in Veldman states as follows: "these findings demonstrate that T cell recognition of distinct Dsg3 peptides is restricted by distinct HLA class II molecules and is independent from the development of Pemphigus vulgaris." This sentence, only refers to the observation that the types of Dsg3 peptides that are recognized by T-cells do not change as the severity of PV progresses, and not, as alleged in the Office Action, that Dsg3 peptides themselves are not involved in the disease.

The authors in Veldman found no evidence of a first set of peptides being associated primarily with mild PV and a second set of different peptides associated with severe PV, and this finding is communicated in the disputed sentence. This finding contrasts with other autoimmune diseases where the types of epitopes recognized by the immune system evolve during the development of the disease in a phenomenon called epitope spreading. Epitope spreading, and its absence in PV patients, is discussed on page 3890, 2nd column, 1st full paragraph of Veldman, which states as follows: "[o]ur findings suggests that intramolecular epitope spreading of Dsg3 T cell epitopes does not occur once the disease is clinically apparent since there was no direct relationship between Dsg3 peptide reactivity and a distinct clinical phenotype (*i.e.* active vs. remittent disease)." Again, the disputed sentence refers to the lack of epitope spreading in PV, *i.e.* the development of PV is independent of the types of peptides that are recognized.

Applicants respectfully submit that the sections of Veldman cited by in the Office Action

fail to support a case of nonenablement, not just because Veldman fails to teach the peptide of claim 1, but because the presence or absence of epitope spreading is an irrelevant inquiry to enablement.

(III) Subsequent Work by the Authors in Veldman Provides a Mechanism for Dsg3 peptides in PV Development

Not only does Veldman fail to undermine the therapeutic benefit of the claimed peptides, subsequent work from several of the authors of Veldman, including Christian Veldman, provides a mechanistic basis for the effectiveness of a Dsg3 peptide to treat PV (see Veldman *et al.* (2004) *J. Immunol.*; 172(10): 6468-75; “Veldman II”, **Exhibit A**).

Consistent with the importance of immune responses against Dsg3 in PV subjects, Veldman II teaches on page 6471, 2nd column, 1st paragraph, that as compared to PV patients, “none of the healthy donors had autoantibodies against Dsg3 as determined by ELISA”. Veldman II teaches that even though both normal and PV subjects have Th1 cells that recognize PV peptides, other types of T cells (Tc1 regulatory cells) that also bind Dsg3 peptides are differentially found in normal vs. PV subjects, and can serve as a basis for presence of the Dsg3 autoantibodies in PV patients. For example, page 6473, 1st column, last paragraph, states that “[t]he predominant isolation of the Dsg3-specific Tr1 cells from the peripheral blood of healthy donors strongly suggest that these Tr1 cells may be involved in the maintenance of self tolerance against Dsg3.” Veldman II, therefore, provides a mechanistic basis as to how a Dsg3 peptide may be used as a therapeutic for PV.

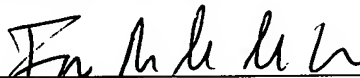
Based on the amendment to claim 1 and the arguments set forth above, Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

CONCLUSIONS

Applicant believes no fee is due with this response under than the \$395 fee for filing an RCE and a \$285 fee for a three-month extension of time, *i.e.* a two-month extension of time fee was paid previously, so the fee due at this time corresponds to \$510 (3-month fee) minus \$225 (2-month fee). However, if any additional fees are due, please charge our Deposit Account No. 18-1945, under Order No. PEPT-P01-005 from which the undersigned is authorized to draw.

Dated: September 30, 2005

Respectfully submitted,

By 

Ignacio Perez de la Cruz

Registration No.: 55,535

ROPES & GRAY LLP

One International Place

Boston, Massachusetts 02110-2624

(617) 951-7000

(617) 951-7050 (Fax)

Attorneys/Agents For Applicant

Type I Regulatory T Cells Specific for Desmoglein 3 Are More Frequently Detected in Healthy Individuals than in Patients with Pemphigus Vulgaris¹

Christian Veldman, Annette Höhne, Detlef Dieckmann, Gerold Schuler, and Michael Hertl²

Pemphigus vulgaris (PV) is a severe autoimmune bullous skin disorder and is primarily associated with circulating autoantibodies against desmoglein 3 (Dsg3) that are presumably regulated by Th cells. The aim of this study was to identify Dsg3-specific T regulatory (Tr) cells that may help to maintain and restore natural tolerance against Dsg3. Dsg3-responsive IL-10-secreting Tr1 cells were isolated by MACS cytokine secretion assay from healthy carriers of the PV-associated HLA class II alleles, DRB1*0402 and DQB1*0503, but were only rarely detected in PV patients. The Dsg3-specific Tr1 cells secreted IL-10, TGF- β , and IL-5 upon Ag stimulation, proliferated in response to IL-2 but not to Dsg3 or mitogenic stimuli, and inhibited the proliferative response of Dsg3- and tetanus toxoid-responsive Th clones in an Ag-specific (Dsg3) and cell number-dependent manner. Moreover, their inhibitory effect was blocked by Ab against IL-10, TGF- β , and by paraformaldehyde fixation. These observations strongly suggest that 1) Dsg3-responsive Tr1 cells predominate in healthy individuals, 2) their growth requires the presence of IL-2, and 3) they exert their Dsg3-dependent inhibitory function by the secretion of IL-10 and TGF- β . Because autoaggressive T cells responsive to identical epitopes of Dsg3 were recently found both in PV patients and healthy individuals, the identified Tr1 cells may be critically involved in the maintenance and restoration of tolerance against Dsg3. *The Journal of Immunology*, 2004, 172: 6468–6475.

Pemphigus encompasses a group of life-threatening autoimmune bullous diseases characterized by intraepithelial blister formation caused by loss of adhesion between keratinocytes induced by autoantibodies directed against desmosomal adhesion proteins (1). Pemphigus vulgaris (PV),³ the most severe variant, is characterized primarily by mucosal lesions (generally when IgG autoantibodies against desmoglein 3 (autoantigen of PV) (Dsg3) are present), and mucocutaneous blisters and erosions (when IgG autoantibodies against both, Dsg3 and desmoglein 1 (autoantigen of pemphigus foliaceus) (Dsg1), are present) (1, 2). Autoantibody production in PV is polyclonal and most autoantibodies are of the IgG4 subclass in patients with active disease while autoantibodies of both IgG1 and IgG4 subtypes are predominant in chronic PV (2). The pathogenic role of autoantibodies against Dsg1 and Dsg3 has been clearly established despite the identification of potential additional autoantigens. The transfer of purified autoantibodies into neonatal mice leads to the in vivo formation of intraepidermal blisters (2). Moreover, Dsg3-deficient

mice express a phenotype with mucosal blisters/erosions that clearly resembles the clinical findings seen in PV (3).

Involvement of Th cells in the pathogenesis of PV has been suggested by several epidemiological studies showing that HLA-DRB1*0402 is associated with PV in Jewish and HLA-DQB1*0503 in non-Jewish populations (2). Both Dsg3-reactive Th1 and Th2 cells were identified by our group and others (4, reviewed in Ref. 5) and appear to recognize epitopes of the extracellular domain of Dsg3 in association with HLA-DRB1*0402 and HLA-DQB1*0503 (6). A critical role for autoreactive T cells in the induction and regulation of Dsg3 autoantibody production has been suggested by a recent study by Nishifuji et al. (reviewed in Ref. 5). Anti-Dsg3 autoantibodies secreted by autoreactive B cells were detected by means of ELISPOT assay upon in vitro stimulation of peripheral lymphocytes from PV patients with Dsg3. In contrast, activation of autoreactive B cells was virtually absent upon depletion of the peripheral lymphocytes from CD4⁺ T cells. It is noteworthy that autoreactive Th cells recognizing identical epitopes of the Dsg3 ectodomain were also identified in healthy individuals that express the PV-associated HLA class II alleles (4–6). These findings suggest that PV is the consequence of a loss of natural tolerance against Dsg3 on the B cell level. Active immune regulation may thus be operative in Dsg3-responsive healthy individuals. It is presumed that this is not the consequence of deletion or immune deviation of autoreactive T cells because autoreactive Th cells specific for identical Dsg3 epitopes were detected both in patients and healthy donors (6).

There is now compelling evidence that CD4⁺ T cells, specialized in suppressing immune responses, play a critical role in immune regulation. Three major populations of T regulatory (Tr) cells have been identified based on their distinct phenotype (CD4⁺CD25⁺) or cytokine secretion pattern (Tr1 and Th3 cells). Although the CD4⁺CD25⁺ subset mediates suppression in a non-Ag-specific manner, the later Tr cell types may act in an Ag-specific way. Tr1 cells can be distinguished from Th3 cells because the former preferentially exert their regulatory effects via production

Department of Dermatology, University of Erlangen-Nürnberg, Erlangen, Germany
Received for publication January 9, 2004. Accepted for publication March 8, 2004.

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¹ This study was supported by grants from the German Research Society (Deutsche Forschungsgemeinschaft; He 1602/6-1, to M.H.), the Interdisciplinary Center for Clinical Research (C9, to M.H.), and the Erlanger Leistungsbezogene Anschubfinanzierung und Nachwuchsförderung Fonds (00.03.22.1, to M.H., and 01.09.19.1, to C.V.), University of Erlangen, Germany.

² Address correspondence and reprint requests to Dr. Michael Hertl, Department of Dermatology, University of Erlangen-Nürnberg, Hartmannstrasse 14, D-91054 Erlangen, Germany. E-mail address: Michael.Hertl@derma.med.uni-erlangen.de

³ Abbreviations used in this paper: PV, pemphigus vulgaris; Dsg1, desmoglein 1 (autoantigen of pemphigus foliaceus); Dsg3, desmoglein 3 (autoantigen of PV); Tr, T regulatory; TCC, T cell clone; Ni, nickel; SI, stimulation index; G1TR, glucocorticoid-induced TNFR; TT, tetanus toxoid.

of IL-10 (7), while Th3 cells preferentially secrete the immunosuppressive cytokine, TGF- β . Tr1 cells exist naturally in the human mucosa and maintain intestinal homeostasis against bacterial pathogens (8) and parasites (9) via the production of IL-10 and TGF- β . Similarly, MHC-autoreactive Tr1-like T cell clones (TCC) isolated from the peripheral blood of healthy donors suppressed Ag-specific T cell responses by the secretion of IL-10 and TGF- β (10).

There is evidence that Tr1 cells may indeed act in an Ag-specific manner. In nickel (Ni) allergy, nonallergic subjects carry Ni-specific T cells that fulfill the criteria of Tr1 cells based on their cytokine profile (IL-10, IL-5, IFN- γ , low IL-4) and their ability to suppress the proliferative response of Ni-activated Th1 cells (11) and may thus be critically involved in the down-regulation of Ni-specific Th cell responses in vivo. IL-10⁺ Tr cells were also detected in patients allergic to bee venom upon specific immunotherapy with phospholipase A which suppressed the proliferative response of allergen-specific Th cells (12). Moreover, the expression of IL-10 increased during specific immunotherapy with phospholipase A suggesting that the protective effect of this regimen was directly correlated to the presence of IL-10⁺ allergen-specific Tr cells.

In this study, we assessed whether the presence or absence of Dsg3-specific Tr1 cells in Dsg3-responsive healthy donors and PV patients, respectively, may be one explanation for the development of tolerance vs autoimmunity against Dsg3. In fact, Dsg3-reactive

IL-10-secreting Tr1 cells were identified in five of six healthy carriers of PV-associated HLA class II alleles (80%) and only in 2 of 12 PV patients (17%) that suppressed the proliferative response of Dsg3-reactive Th cells in an Ag-specific and cytokine (IL-10/TGF- β) dependent manner. In addition, 50% of the isolated IL-10⁺ TCC from the healthy donors were of the Tr1 type while only 16% of the IL-10⁺ TCC from the PV patients were of the Tr1 type. These findings suggest that Dsg3-specific Tr may be involved in the maintenance of peripheral tolerance to Dsg3 in healthy individuals and in the restoration of tolerance against Dsg3 in PV patients.

Materials and Methods

Patients and controls

Heparinized blood samples (60 ml) were obtained from a total of 14 adult patients, in treatment at the Dermatology Department (University of Erlangen, Erlangen, Germany), with active and remittent PV as well as from 11 healthy control individuals. All PV patients and healthy control donors gave written consent to participate in this study. The clinical diagnosis of PV was confirmed by 1) histopathology (suprabasal acantholytic blisters), 2) direct immunofluorescence microscopy (epidermal intercellular IgG and/or C3 deposits in perilesional skin), and 3) the detection of circulating autoantibodies by indirect immunofluorescence microscopy (intercellular IgG binding to epithelial cells of monkey esophagus) and/or by a commercial Dsg3-ELISA (MDL, Naka-ku Nagoya, Japan) (Table I). PV was defined to be active for patients suffering from blisters/erosions on the mucosal surfaces and/or skin; some of these patients had already received immunosuppressive treatment (Table I). Patients with remittent PV had not

Table I. Clinical and immunological profile of the studied patients with PV

Clinical Status ^a	Patient	HLA Class II ^b Alleles		Clinical Phenotype ^c		Medication ^d (per day)	Autoantibody Profile ^e (IgG)	IL-10 ⁺ T Cell Clones ^f	
		DRB1	DQB1	Skin	Mucosa		Anti-Dsg3	Th2	Tr1
Active PV	PV2	0401, 1401	0301, 0503	None	Discrete erosions	24 mg of MP	8	<i>n</i> = 6 (P2-6, P2-8, P2-11, P2-12, P2-18, P2-26)	
	PV3	0804, 1411	0402, 0503	Diss. blisters	Erosions	None	1		
	PV4	0701, 1401	02, 0503	None	Discrete oral erosions	None	58		<i>n</i> = 1 (P6-7)
	PV6	0405, 1401	0302, 0503	None	Discrete erosions	20 mg of LF	181	<i>n</i> = 3 (P6-6, P6-10, P6-12)	
	PV7	0402, 0405	02, 0302	None	Erosions	2 g of MPM, 7.5 mg of PD	217		
	PV8	0402, 1104	0301, 0302	None	Buccal erosions	None	2		
	PV9	0402, 1104	0301, 0302	None	Gingival erosions	None	168		
	PV10	0803, 1405	0503, 0601	Blisters/erosions	Erosions	100 mg of AZA, 24 mg of MP	203		
	PV11	0402, 1104	0301, 0302	Discrete erosions	Buccal erosions	100 mg of AZA, 24 mg of MP	154	<i>n</i> = 3 (P11-4, P11-10, P11-12)	
	PV13	1401, 1502	0503, 0601	None	Single erosions	None	248		
	PV14	0301, 0402	02, 0302	Discrete augural erosions	None	100 mg of AZA	38	<i>n</i> = 4 (P14-8, P14-10, P14-19, P14-25)	<i>n</i> = 2 (P14-16, P14-18)
Remittent PV	PV1	0101, 1401	0501, 0503	None (none)	None (buccal erosions)	None	30		
	PV5	0401, 0804	0302, 0402	None (few blisters on the trunk)	None (oral erosions)	None	6		
	PV12	0402, 1301	0302, 0603	None (crusty erosions of the scalp)	None (discrete oral erosions)	None	42		

^a As classified in *Materials and Methods*.

^b PV-associated HLA class II alleles (bold).

^c At the time of study; the clinical description of mucosal or cutaneous lesions in parentheses refers to the initial clinical phenotype.

^d AZA, azathioprine; LF, leflunomide; MP, methyl prednisolone; MPM, mycophenolate mofetil; PD, prednisolone.

^e As determined by ELISA with Dsg3. Values are expressed as index value ($(A_{405}(\text{Sample}) - A_{405}(\text{negative control})) / (A_{405}(\text{positive control}) - A_{405}(\text{negative control})) \times 100$). Index values >14 were considered to be significant.

^f IL-10⁺ TCC isolated from each patient (Th2; Tr1).

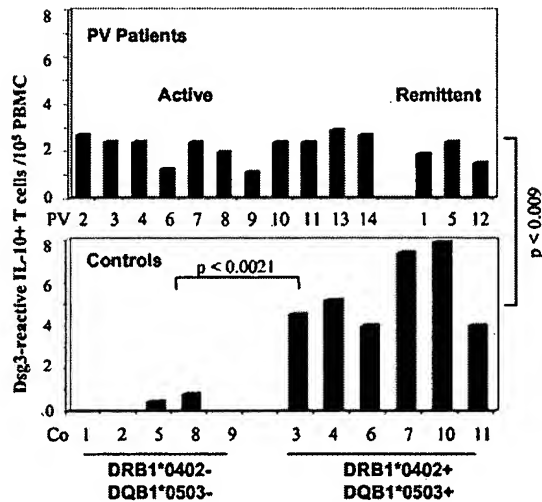


FIGURE 1. Frequency analysis of desmoglein 3-reactive IL-10⁺ T cells in patients with PV and healthy donors. PBMC from 14 PV patients and 11 healthy donors (controls) were stimulated with Dsg3 for 16 h and IL-10⁺ T cells were isolated by MACS secretion assay. Both PV patients and healthy carriers of the PV-associated HLA class II alleles, HLA-DRB1*0402 and DQB1*0503, mounted significant IL-10⁺ T cell responses against Dsg3. In contrast, healthy carriers of other HLA class II alleles did not. The frequency of the IL-10⁺ T cells in healthy carriers of the PV-associated HLA class II alleles was significantly higher compared with those in the PV patients ($p < 0.009$) and the other healthy donors ($p < 0.0021$).

experienced new mucosal blisters/erosions for six or more months before the study (Table I). HLA class II genotyping was performed in all the patients and controls. The determination of HLA-DRB1 and DQB1 alleles was conducted at high resolution by enzyme-linked probe-hybridization assay (Biotest, Dreieich, Germany) using locus-specific PCR products as templates (Dr. R. Wassmuth, Institute for Transplantation Diagnostics and Cell Therapeutics, Düsseldorf University Medical Center, Düsseldorf, Germany).

Production and purification of human rDsg3

The recombinant protein PVhis, a fusion protein consisting of the entire extracellular domain of Dsg3 linked to an E tag and histidine tag was used as a source of human Dsg3 and was expressed in a baculovirus system with SF21 insect cells as described previously (4, 6). For the production of Dsg3

protein, 3×10^8 High-Five insect cells were inoculated with PVhis baculovirus at a multiplicity of infection of 10. Culture supernatants of baculovirus-infected insect cells were collected after 4 days and Dsg3 protein was purified from culture supernatants over Ni-NTA-linked agarose (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Ex vivo isolation and quantification of Dsg3-reactive IL-10-secreting T cells

IL-10-secreting T cells were isolated from short-term (16 h) cultures with $6-9 \times 10^7$ PBMC of PV patients/controls and 10 $\mu\text{g/ml}$ Dsg3 by MACS cytokine secretion assay following the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Upon termination of the cultures, high affinity anti-human IL-10 Ab which bound to the surface of the cytokine-secreting T cells was added. Labeling with a secondary magnetic bead-coupled Ab allowed for specific enrichment of IL-10-secreting T cells by passage over magnetic columns that were finally counted in a hemocytometer, as recently described for the isolation of IL-4- and IFN- γ -secreting Dsg3-responsive Th cells (4). The number of MACS-isolated T cells was divided by the total number of PBMC to obtain the frequency of IL-10⁺ T cells per 10^5 PBMC. The statistical software package SAS (version 8.2; SAS Institute, Cary, NC) was used for descriptive uni- and bivariate statistics.

In vitro propagation of IL-10-secreting T cells

Following isolation by MACS assay, Dsg3-reactive IL-10-secreting T cells were cloned by limiting dilution and were expanded by repeated stimulation with 1% PHA (Life Technologies, Karlsruhe, Germany) and x-irradiated (50 Gy) allogenic PBMC as APC followed by addition of IL-2 (10 U/ml; BD-Boehringer, Heidelberg, Germany) as described recently (4). For proliferative assays, human T cells were cultured in a medium consisting of RPMI 1640 (Life Technologies) with 10% heat-inactivated pooled human serum (Life Technologies), 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin (P/S), and 20 mM L-glutamine (L-Glu). Cloned T cells (5×10^4) were cultured in duplicate with Dsg3 (10 $\mu\text{g/ml}$) or PHA (1%; Sigma-Aldrich, Taufkirchen, Germany) and 5×10^4 x-irradiated (50 Gy) autologous PBMC as APC in 200- μl 96-well round-bottom microtiter plates (BD-Falcon, Heidelberg, Germany) for 72 h at 37°C in 5% CO₂. In addition, TCC were also stimulated with plate-bound anti-CD3 (clone UCHT1; at 10 $\mu\text{g/ml}$) plus soluble anti-CD28 (clone CD28.2; at 10 $\mu\text{g/ml}$) (both from BD PharMingen, Heidelberg, Germany). T cell proliferation was determined by the extent of incorporation of [³H]thymidine (Dupont, Mechelen, Belgium), which was added for the final 18 h of the culture and was expressed as a stimulation index (SI), which is the ratio of [³H]thymidine uptake (cpm) in cultures with Ag to the uptake in cultures without Ag; an SI ≥ 3 was considered to represent significant stimulation.

Cytokine profile of IL-10⁺ T cells

TCC were stimulated with Dsg3 and autologous x-irradiated (50 Gy) PBMC as APC or anti-CD3/anti-CD28 for 48 h and culture supernatants

Table II. HLA class II alleles of healthy individuals and derived Dsg3-specific IL-10⁺ T cell clones

Controls	HLA-DRB1 ^a	HLA-DQB1 ^a	IL-10 ⁺ T Cell Clones ^b	
			Th2	Tr1
1	1302, 1501	0602, 0604		
2	0301, 0401	02, 0302		
3	0402 , 1401	0302, 0503	$n = 3$ (C3-13, C3-19, C3-25)	$n = 3$ (C3-6, C3-14, C3-21)
4	1401, 1501	0503 , 0602	$n = 2$ (C4-4, C4-11)	$n = 5$ (C4-2, C4-10, C4-23 C4-28, C4-40)
5	1501, -	0602, -		
6	0402 , 1501	0303, 0602	$n = 6$ (C6-5, C6-13, C6-31, C6-33, C6-39, C6-42)	$n = 2$ (C6-9, C6-34)
7	0402 , 1102	0301, 0302	$n = 3$ (C7-17, C7-22, C7-42)	$n = 4$ (C7-19, C7-27, C7-32, C7-34)
8	0101, 1501	0501, 0602		
9	0701, -	02, -		
10	1104, 1401	0301, 0503	$n = 1$ (C10-6)	$n = 1$ (C10-2)
11	1301, 1401	0503 , 0603		

^a PV-associated HLA class II alleles (bold).

^b IL-10⁺ T cell clones derived from each donor.

were analyzed by ELISA for TGF- β immunoreactivity according to the manufacturer's (BD Pharmingen) instructions and by cytometric bead array for IL-2, IL-4, IL-5, IL-10, IFN- γ , and TNF- α immune reactivities (Th1/Th2 Cytokine CBA 1; BD Pharmingen) according to the manufacturer's instructions.

Flow cytometric analysis

To further characterize the IL-10-secreting TCC, their expression of various surface molecules was compared with Dsg3-specific Th2-like TCC. T cells were immunostained 14–20 days after *in vitro* stimulation with Dsg3 or PHA with the following Ab: PE- and FITC-conjugated Ab against CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD19 (HIB19), CD25 (M-A251), CD45RO (UCHL 1), CTLA-4 (BN13.1), CD122 (MiK- β 2), HLA-DR (G46-6), CCR4 (1G1), CCR5 (2D7), CCR7 (3D12; all from BD Pharmingen), TGF- β (TB21; IQ Products, Groningen, The Netherlands), glucocorticoid-induced TNFR (GITR) (N-14; Santa Cruz Biotechnology, Heidelberg, Germany), and respective mouse and rat isotype controls were used. Cells were washed and stained for 30 min at room temperature with optimal dilutions of each Ab, washed again, and analyzed by flow cytometry (FACS Scan and CellQuest Software; BD Biosciences, Heidelberg, Germany).

In vitro regulatory function of IL-10⁺ T cells

IL-10⁺ TCC were cocultured with HLA-matched autologous Dsg3- and tetanus toxoid (TT)-specific Th2 clones, Dsg3 (10 μ g/ml) and autologous, x-irradiated (50 Gy) PBMC as APC. The proliferative response of the "responder" Th2 clones to Dsg3 and the potential extent of inhibition of Th cell proliferation by the IL-10⁺ TCC was determined by the uptake of [³H]thymidine. Blocking experiments used to investigate whether the regulatory function of the IL-10-secreting T cells was mediated primarily by soluble factors or required cell-cell contact were also performed. Unconjugated anti-IL-10 (JES3-19F1; 10 μ g/ml) and anti-TGF- β (A75-2.1; 2 μ g/ml; both BD Pharmingen) were added to the cultures of responder TCC and IL-10⁺ TCC cells and their effect was measured by the uptake of [³H]thymidine after 2–3 days. To address the critical role of additional suppressive factors secreted by the Tr1 cells, the IL-10⁺ TCC were also fixed with 2% paraformaldehyde for 1 h at 4°C and were subsequently thoroughly washed before use in coculture experiments with responder TCC.

In transwell experiments, a total of 10⁵ cloned Dsg3-responsive Th cells were stimulated with 10⁵ autologous, x-irradiated (50 Gy) PBMC as APC and Dsg3 in 24-well plates; in addition, 2 \times 10⁴ Tr cells (1:5) were either added directly to the cultures or 5 \times 10⁵ cloned Th cells and 10⁵ Tr cells were placed in transwell chambers (Millicell, 0.4 μ m; Millipore, Schwalbach, Germany). After 3 days of coculture, T cells were transferred to 96-well plates (5 \times 10⁴ cells/well) in triplicate and T cell proliferation was determined by the extent of incorporation of [³H]thymidine, which was added for the final 18 h of the culture.

Results

Frequency analysis of Dsg3-reactive IL-10⁺ T cells in PV patients and healthy donors

The majority (13 of 14) of the studied PV patients expressed either HLA-DRB1*0402 (43%) or HLA-DQB1*0503 (50%), HLA class II alleles prevalent in PV (Table I). Independent of the clinical activity and the immunosuppressive treatment of PV, all of the PV patients exhibited low but reproducibly detectable frequencies of IL-10⁺, Dsg3-responsive T cells ($2.2 \pm 0.5/10^5$ cells; Fig. 1). In addition, 11 healthy donors were studied, 6 of whom were either positive for HLA-DRB1*0402 ($n = 3$) and/or HLA-DQB1*0503 ($n = 4$; Table II); none of the healthy donors had autoantibodies against Dsg3 as determined by ELISA (not shown). All of the healthy individuals expressing either HLA-DRB1*0402 or HLA-DQB1*0503 carried IL-10⁺ Dsg3-responsive T cells which were detected at significantly higher frequencies ($5.5 \pm 1.7/10^5$ PBMC) than that observed for PV patients ($2.2 \pm 0.9/10^5$ PBMC; $p < 0.0009$; Fig. 1). Frequencies of IL-10⁺ Dsg3-responsive T cells of PV patients were similar to the frequencies of IL-4⁺ Dsg3-responsive T cells reported in a previous study (13). In contrast, Th2 frequencies of healthy donors in the same study were below the detection limit (13). It is noteworthy that none of the healthy carriers of PV-unrelated HLA class II alleles exhibited Dsg3-specific IL-10-secreting T cell responses (Fig. 1).

In vitro expansion of IL-10⁺ T cell clones

Dsg3-reactive IL-10-secreting T cells were isolated by MACS secretion assay, cloned by limiting dilution and were expanded by repeated stimulation cycles with 1% PHA, 10 U/ml IL-2, and allogeneic PBMC as APC. Altogether, a total of 49 IL-10⁺ Dsg3-specific TCC were derived from four PV patients ($n = 19$; Table I) and from five healthy individuals ($n = 30$; Table II). Of the 19 IL-10⁺ TCC derived from the PV patients, 16 (84%) were of the Th2 type and 3 (16%) were of the Tr1 type.

In contrast, 50% of the 30 IL-10⁺ TCC from the healthy donors were of the Tr1 type. IL-10⁺ Th2 cells were CD4/CD45RO⁺, GITR⁺, membrane TGF- β ⁺, produced Th2 cytokines, and proliferated in response to Dsg3 (Table III, Fig. 2). In contrast, the Tr1 cells were CD4/CD45RO⁺, GITR⁺, membrane TGF- β ⁺, secreted IL-10, IL-5, TGF- β but no IL-4, and did not proliferate in response to Dsg3 (Table III, Fig. 2) (7, 14).

Table III. Cytokine profile of Dsg3-specific IL-10⁺ T cell clones^a

TCC	IL-2 ^a Ag/Mit	IL-4 ^a Ag/Mit	IL-5 ^a Ag/Mit	IL-10 ^a Ag/Mit	TNF- α ^a Ag/Mit	IFN- γ ^a Ag/Mit	TGF- β ^a Ag/Mit	Dsg3 (SI) ^a	Anti-CD3/28 (SI) ^a
Tr1^b									
C4-4	-/-	-/+	(+)/(+)	+/-	+/-	-/+	+/-	1	1.1
C4-11	-/-	-/-	-/+	+/-	+/-	-/+	+/-	1.1	1.1
C6-13	-/-	-/-	+/-	+/-	+/-	-/+	+/-	1.1	1
C6-31	-/-	-/-	+/-	+/-	+/-	+/-	+/-	1	1
C7-42	-/-	-/-	+/-	+/-	+/-	-/+	+/(+)	1.1	1.1
P14-16	-/-	-/-	-/+	+/-	+/-	-/+	+/-	1	1.1
Th2^c									
C4-10	-/-	+/-	+/-	+/-	-/-	-/-	-/-	3.3	4.2
C4-28	-/-	-/-	+/-	+/-	-/-	-/-	-/-	4.3	4.3
C6-9	-/-	+/-	+/-	+/-	-/-	-/-	-/-	2.7	3.3
C6-34	-/-	+/-	+/-	+/-	-/-	-/-	-/-	3.2	4.1
C7-19	-/-	+/-	+/-	+/-	-/-	-/-	-/-	3.4	3.7

^a Upon stimulation with Dsg3/anti-CD3/CD28; SI (cpm with Ag/cpm without Ag).

^b IL-2 (Dsg3/anti-CD3/CD28): 0/0–61; IL-4: 0/35–128; IL-5: 343–646/898–5765; IL-10: 11–49/0; TNF- α : 166–307/233–489; IFN- γ : 0/134–902; TGF- β : 101–478/250–446 pg/ml.

^c IL-2 (Dsg3/anti-CD3/CD28): 276–704/367–734; IL-4: 37–125/37–138; IL-5: 46–2017/43–1334; IL-10: 21–217/0–11; TNF- α : 0/0; IFN- γ : 0/0; TGF- β : 0/0 pg/ml.

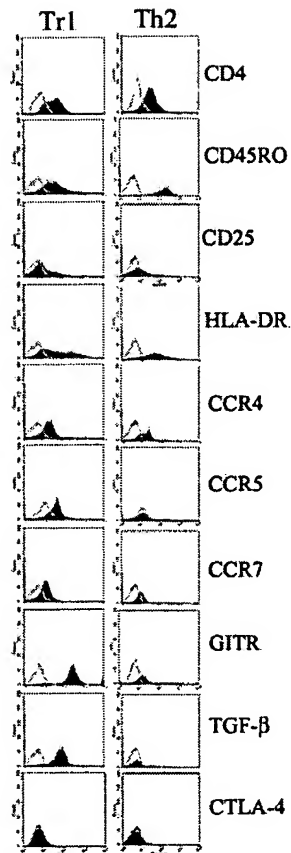


FIGURE 2. IL-10⁺ Tr1-like clones exhibit distinct phenotypic differences when compared with IL-10⁺ Th2 clones. Two distinct T cell populations were identified. The first CD3⁺CD4⁺, memory (CD45RO⁺) T cell population expressed HLA-DR, CCR4, CCR5, CCR7, and high quantities of GITR and membrane-bound TGF-β and was accordingly classified as Tr1 cells (7). The second population, which was also CD3⁺CD4⁺CD45RO⁺, expressed HLA-DR and CCR4, but showed no or only marginal expression of CD25, CCR5, CCR7, GITR, and membrane-bound TGF-β. This population was thus classified as Th2 cells (14). These findings were seen with a total of eight Tr1- and six Th2-like TCC.

Phenotypic analysis of IL-10⁺ T cells

FACS analysis on the two distinct IL-10⁺ T cell populations showed a differential expression pattern of distinct surface markers (Fig. 2). The first CD3⁺CD4⁺, memory (CD45RO⁺) T cell population was negative for CD8, CD14, CD19, and CTLA-4 and expressed low CD25, substantial HLA-DR, CCR4, CCR5, CCR7, and strong GITR and membrane-bound TGF-β. This population

was accordingly classified as Tr1 cells (7). The second population, which was also CD3⁺CD4⁺CD45RO⁺, was negative for CD8, CD14, CD19, and CTLA-4, expressed HLA-DR and CCR4, but showed no or only marginal expression of CD25, CCR5, CCR7, GITR, and membrane-bound TGF-β. This population was thus classified as Th2 cells (14). These findings were seen with a total of eight Tr1- and six Th2-like TCC.

Cytokine profile of IL-10⁺ T cells

The IL-10⁺ TCC were stimulated either with Dsg3 and HLA-matched, x-irradiated PBMC as APC or anti-CD3/anti-CD28 for 48 h and culture supernatants were analyzed by ELISA (TGF-β) and cytometric bead array (IL-2, IL-4, IL-5, IL-10, TNF-α). As shown in Table III, two distinct T cell populations were identified. A subset of the IL-10⁺ TCC failed to proliferate in response to Dsg3 stimulation and produced IL-5, IL-10, TNF-α, and variable quantities of TGF-β upon challenge with Dsg3, resembling Tr1 cells (7). Nonspecific stimulation with anti-CD3/anti-CD28 of the Tr1 cells led to the secretion of IL-5, IFN-γ, TNF-α, and low levels of TGF-β. In contrast, another subset of the IL-10⁺ TCC which showed a proliferative response to Dsg3 produced substantial quantities of IL-4, IL-5, and IL-10 but no Th1 cytokines upon stimulation with Dsg3 or anti-CD3/CD28 (Table III), resembling Th2 cells (14). Upon stimulation with Dsg3, the Tr1 clones C4-4, C6-13, C7-42 produced IL-10 (25, 40, 23 pg/ml), TGF-β (189, 187, 463 pg/ml), IL-5 (588, 430, 403 pg/ml), and no IL-4 (<10 pg/ml), while the Th2 clones C4-28, C6-34, C6-9, C7-19 produced IL-10 (62, 101, 92, 113 pg/ml), IL-4 (46, 87, 63, 103 pg/ml), IL-5 (267, 545, 633, 329 pg/ml) and no TGF-β (<10 pg/ml).

Proliferative capacity of IL-10-secreting T cells

A subset of the IL-10⁺ TCC that was classified as Tr1 cells based on their cytokine production and phenotype showed no or only little proliferative response to stimulation with Dsg3, PHA, or anti-CD3/CD28 and proliferated only in response to IL-2 (Fig. 3A). In contrast, the Dsg3-responsive IL-10⁺ Th2 clones showed a significant response to Dsg3 and mitogenic stimulation which was augmented by the addition of IL-2 (Fig. 3B).

IL-10⁺ Tr1 cells suppress the proliferative response of Dsg3- and TT-specific Th clones

To analyze the potential regulatory properties of the IL-10⁺ Tr1 cells, coculture experiments with Dsg3- and TT-specific responder T helper clones were performed (Fig. 4). Upon in vitro stimulation with Dsg3, the Tr1 clones significantly inhibited the proliferative response of Dsg3- (Fig. 4A) and TT-responsive (Fig. 4B) TCC.

FIGURE 3. Differential proliferative capacity of IL-10⁺ Tr1 and Th2 clones. The Tr1 clone C4-4 and the Th2 clone C4-28 were cocultured in vitro with Dsg3, 1% PHA, or plate-bound anti-CD3 plus soluble anti-CD28 and x-irradiated PBMC as APC. C4-4 showed virtually no proliferative response to antigenic or mitogenic stimuli as determined by the uptake of [³H]thymidine, while exogenous IL-2 (10 μg/ml) induced a significant proliferative response of C4-4 (A). In contrast, the Dsg3-specific Th2 clone C4-28 showed a vigorous proliferative response to Dsg3, PHA, and anti-CD3/CD28, which was augmented by the addition of IL-2 (10 U/ml) (B). These findings were seen with a total of three Tr1- and seven Th2-like TCC.

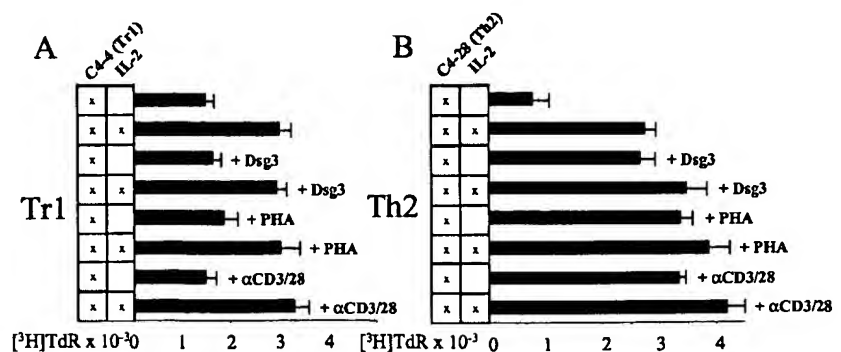
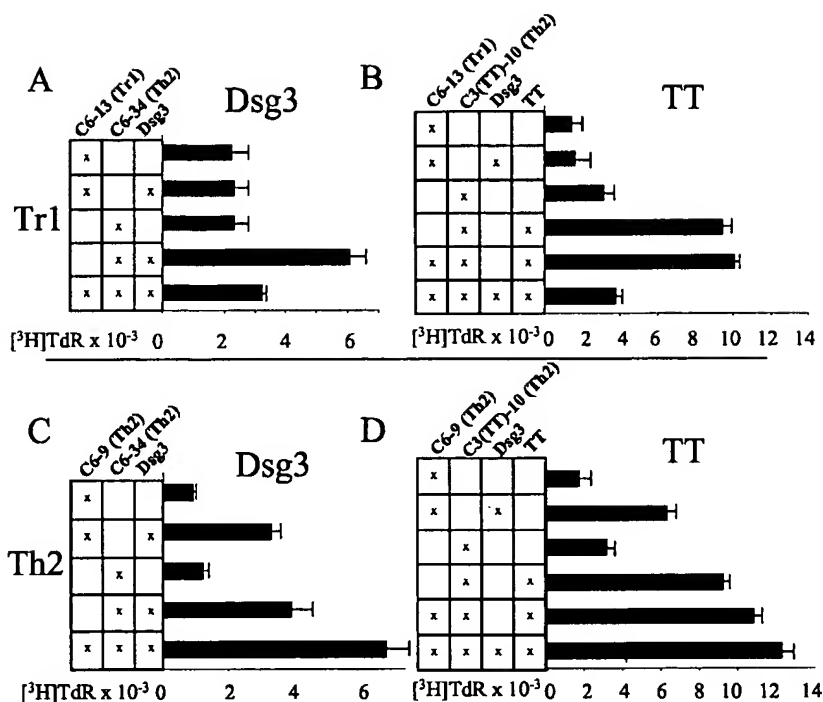


FIGURE 4. Differential inhibitory function of Dsg3-responsive IL-10⁺ Tr1 and Th2 cells. The IL-10⁺ Tr1 clone C6-13 (*A* and *B*) and the Th2 clone C6-9 (*C* and *D*) were cocultured with Dsg3- (*A* and *C*) or TT- (*B* and *D*) specific Th2 clones (C6-34, C3(TT)-10), Dsg3 (10 μ g/ml) and autologous x-irradiated (50 Gy) PBMC as APC. The proliferative response of the TCC to Dsg3 was determined by the uptake of [³H]thymidine. The proliferative response to Dsg3 of the responder TCC C6-34 was strongly inhibited upon coculture with TCC C6-13 (*A*) but not C6-9 (*C*). Accordingly, the TT-dependent proliferation of the TCC C3(TT)-10 was inhibited upon Dsg3-dependent activation of the Tr1 clone C6-13 but not by the Th2 clone C6-9. These findings were seen with a total of six Tr1- and three Th2-like TCC.



This inhibitory effect was cell number-dependent and was detectable up to a Tr1/Th ratio of 1:10 (Fig. 5, *A* and *B*); in these experiments, 5×10^4 responder Th cells were cocultured with variable numbers (2.5×10^3 – 5×10^4) of Tr1 cells. In contrast, Dsg3-responsive IL-10⁺ Th2 clones had no inhibitory effect on the proliferative response of Dsg3- (Fig. 4*C*) and TT-specific (Fig. 4*D*) Th clones.

Next, we investigated whether the regulatory function of the IL-10⁺ Tr1 cells was mediated by soluble factors or required cell-cell contact. The inhibitory effect of the IL-10⁺ Tr1 clones on the proliferative response to Dsg3 of a Th2 clone was reversed by blocking Ab against IL-10 and TGF- β and upon fixation of the Tr1 cells (Fig. 5, *C* and *E*). In addition, there was a dose-dependent inhibition of the Dsg3-responsive Th2 clones when exogenous rhIL-10 (1–100 ng/ml) or TGF- β (1–100 ng/ml) was added (not shown). Despite the obvious inhibitory action of soluble factors, the additional requirement for close interaction between regulatory and responder T cells could not be excluded. Separation of the two T cell populations in transwell chambers did not abolish the suppressive effect of the Tr1 clones (Fig. 5, *D* and *F*). These observations suggest that direct cell contact is not essential for the inhibitory capacity of the IL-10⁺ Tr1 cells, as the semipermeable membrane of transwell chambers allowed free passage of soluble factors, but excluded direct cell-cell contact.

Discussion

In this study, Dsg3-responsive, type 1 T regulatory (Tr) cells were preferentially isolated from the peripheral blood of a subset of healthy individuals who carried the PV-associated HLA class II alleles, HLA-DR β 1*0402 and DQ β 1*0503, and only from a minority of patients with PV. The Tr1 cells exhibited a Dsg3-induced inhibitory action on the proliferative response of Dsg3-responsive, autoreactive Th clones (and also TT-responsive Th clones) which was cell-cell contact independent and was mediated by the cytokines, IL-10, and TGF- β . The predominant isolation of the Dsg3-specific Tr1 cells from the peripheral blood of healthy donors strongly suggests that these Tr1 cells may be involved in the maintenance of self tolerance against Dsg3.

The aim of this study was to address the potential role of autoantigen-specific Tr cells in autoimmunity vs self tolerance against Dsg3 by comparing the presence of Dsg3-specific Tr cells in PV patients and healthy donors. Dsg3-specific T cells secreting the immunoregulatory cytokine, IL-10 and to a lesser degree, TGF- β , were isolated by MACS cytokine secretion assay, cloned by limiting dilution and thoroughly characterized. Our findings demonstrate that a significant portion of the Dsg3-specific IL-10⁺ TCC isolated from healthy donors exhibited characteristics of type 1 Tr cells based on their phenotype, cytokine profile, and in vitro regulatory function. A major finding of the present study was the observation that the immunosuppressive cytokines IL-10 and TGF- β , which were secreted by the Dsg3-specific Tr1 cells, were exclusively responsible for their regulatory function.

There is only limited evidence for the involvement of autoantigen-specific Tr1 cells in autoimmunity (13). A decreased frequency of CD4⁺ T cells producing IL-10, but not IL-2 or IL-4, was observed in rheumatoid arthritis, suggesting a defect in down-regulation of T cell tolerance in this disease (15). In the NOD mouse model of diabetes, both autoantigen-reactive Tr1 cells as well as Th2 cells were induced by immunization with two immunodominant glutamic acid decarboxylase 65 peptides (16). Adoptive transfer of the glutamic acid decarboxylase-reactive Tr1 cells into NOD/scid mice prevented the onset of diabetes. Their mode of action, i.e., cytokine- or cell contact-dependent suppression remained unclear. In patients suffering from multiple sclerosis, oral treatment with myelin basic protein and proteolipid protein clinically induced a state of tolerance associated with a significant increase of myelin basic protein- or proteolipid protein-specific T cells, which secreted predominately TGF- β and moderate quantities of IL-4 and IL-10, which were accordingly classified as Th3 cells (17). Based on the differential secretion of TGF- β and IL-10, respectively, Th3 and Tr1 cells presumably represent two different Tr cell subsets (13). IL-10^{-/-} mice develop colitis and are susceptible to a condition resembling rheumatoid arthritis (18), indicating that this cytokine has an essential role both in maintaining intestinal tolerance to normal enteric Ags and in systemic tolerance

as suppressors of inflammation (22). Further studies are required to address the question as to whether activation of the identified Dsg3-specific Tr1 cells through GITR enhances their regulatory capacity or not. Membrane-bound TGF- β may be also an important effector for cell contact-dependent inhibition of CD4⁺CD25⁺ T cells (23). In contrast, CTLA-4, which is also found on CD4⁺CD25⁺ Tr cells (24), was not expressed by the identified Dsg3-specific Tr1 cells. Because the transcription factor Foxp3 (scurfin) is associated with the regulatory function of T cells, it may be involved in the regulation of GITR and CTLA-4 (25–27). In humans, mutations of Foxp3 induce an autoimmune syndrome characterized by polyendocrinopathy and enteropathy (28).

Tr1 cells, such as the IL-10⁺ Dsg3-specific Tr cells identified in the present study, can be induced in vitro by stimulation of naive T cells in the presence of IL-10 and IFN- α (29). They also appear to be induced by repeated Ag stimulation of naive T cells leading to the down-regulation of immune responses following transfer in vitro (30). Similarly, repetitive in vitro stimulation with APC loaded with tumor-associated Ags (31) or in vivo stimulation with superantigen (32, 33) led to the emergence of CD4⁺ T cells that suppressed naive T cell responses via the production of IL-10. Groux et al. (29) reported the induction of both human and murine Tr1 cells upon chronic activation of CD4⁺ T cells in the presence of IL-10. In a mouse model of inflammatory bowel disease, the transfer of as few as 2×10^5 OVA-specific Tr1 cells (which were induced in vivo by oral administration of OVA) prevented the development of colitis (29).

To the best of our knowledge, this is the first study providing evidence supporting the theory that autoantigen-specific Tr1 cells may be relevant in the maintenance of tolerance against a defined human autoantigen. Our observations strongly suggest that immunological tolerance against Dsg3, the autoantigen of PV may be, at least partly, mediated by Dsg3-specific type 1 Tr cells. These findings provide a sound explanation as to why B cell tolerance against Dsg3 exists in healthy individuals who carry autoaggressive T cells reactive to Dsg3 epitopes identical to those recognized by T cells from the PV patients. Thus, Dsg3-responsive Tr1 cells may represent an ideal tool to therapeutically restore Dsg3-specific immune tolerance in PV.

Acknowledgments

We thank Dr. Ralf Wassmuth (Institute for Transplantation Diagnostics and Cell Therapeutics, Düsseldorf University Medical Center, Germany) for the HLA class II typing; Astrid Mainka for her excellent help with the cell culture and preparation of rDsg3; and Dr. Masayuki Amagai (Dermatology, Keio University, Japan) for rPVhis (Dsg3) baculovirus.

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